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(54) Title: **FILTRATION VACUUM PAD**

(57) Abstract: A gasket is disclosed, adapted for use with a multiwell microplate. The gasket is made of a porous material with openings corresponding to the nozzles of a filter plate. The porous material facilitates movement of solutions by application of either positive or negative pressure. The disclosed gasket solves the problem of cross-contamination which may occur between adjacent nozzles on the underside of a filter plate.

FILTRATION VACUUM PAD

Background of the Invention

Field of the Invention

The Field of the Invention relates generally to vacuum pads, seals and gaskets, particularly to a vacuum pad for use with a high throughput filter plate for use with biological and chemical samples.

Description of the Related Art

Life science research laboratories are increasingly discovering the usefulness of multiwell microplates in conducting high-throughput assays. Microplates have become the preferred platform for automated analysis due to their easy handling by commercially available robotic equipment. Microplates which incorporate a membrane filter at the base of the wells are known as filter plates. Filter plates are becoming more widely used as research tools as well. Filter plates allow separation of liquid and solid material quickly and easily and are compatible with microplate robotic equipment. Commonly used applications of filter plates in molecular biological research include trapping of cells. The trapped cells may be either washed or lysed directly on the filter plate. If the cells are lysed, the filter plate facilitates separation of cellular debris from cell lysates. Filter plates may also be used to collect pass-through fractions individually if desired.

Various applications using filter plates provide certain challenges which must be addressed. For example, it is possible that cross-contamination can occur between adjacent nozzles on the underside of a filter plate. Given the extreme sensitivity of certain applications, it becomes necessary to minimize and eliminate any potential cross-contamination. The approach used to solve this problem should be reliable, inexpensive, and compatible with filter plates and microplate robotic equipment.

Summary of the Invention

The present invention is drawn to a porous gasket sheet having intercommunicating pores in a longitudinal direction, said intercommunicating pores having a size effective to permit air to flow therethrough. Optionally, the gasket sheet has an edge adapted to be connected to a vacuum source. Preferably, the gasket sheet is adapted to be secured between a multiwell filter plate and a receiving plate and the porous gasket has holes corresponding to the wells of the multiwell filter plate.

The gasket sheet may be made of any appropriate material known to the skilled artisan. Preferred materials are selected from the group consisting of polypropylene, polyethylene, polyvinylidene fluoride, polytetrafluoroethylene, nylon, polyethersulfone, and ethyl vinyl acetate.

Preferably, the gasket sheet has a thickness of $\frac{1}{16}$ to $\frac{1}{2}$ inch. More preferably, the gasket thickness is $\frac{1}{16}$ inch to $\frac{1}{8}$ inch.

Preferably, the disclosed gasket sheet has 6 to 384 holes. More preferably, the gasket sheet has 96 holes.

The gasket sheet may have an edge adapted to be connected to a vacuum source, wherein each hole has an inner peripheral edge through which air can be absorbed when the vacuum source is activated. In a preferred

embodiment, the gasket has a range of average pore size of 7 to 250 microns. More preferred is a gasket with a range of average pore size of 45-90 microns. A mean pore size of 70 microns is most preferred.

Also encompassed is a multiwell filter plate in combination with a gasket sheet including any of the elements set forth above, said gasket sheet corresponding in size and shape to the perimeter of said multiwell filter plate and having a plurality of holes corresponding to the respective wells of the multiwell filter plate, said gasket sheet being adapted to fit between said multiwell filter plate and a receiving plate. Optionally, each well of the multiwell filter plate comprises a nozzle for application of a vacuum. Optionally, each well of the multiwell filter plate has a membrane filter at its bottom, and the gasket sheet has an edge adapted to be connected to a vacuum source, wherein the pressure below the membrane filter can be reduced by absorbing air through an inner peripheral edge of each hole when activating the vacuum source.

Also encompassed by the present invention is an elution system comprising a multiwell filter plate with a gasket sheet as set forth above, and a receiving plate, wherein the gasket sheet is secured between the multiwell filter plate and the receiving plate, each well has a membrane filter at its bottom, and the gasket sheet has an edge adapted to be connected to a vacuum source, wherein the pressure below the membrane filter can be reduced by absorbing air through an inner peripheral edge of each hole when activating the vacuum source.

Also encompassed is the use of an elution system as set forth above, in a method of treating cells comprising the steps of:

- (a) applying cells to each well of the multiwell filter plate;
- (b) treating the cells in each well with a solution;
- (c) activating the vacuum source to absorb air through each hole of the gasket sheet, wherein the pressure under the membrane filter is reduced; and
- (d) enhancing draining the used solution through the membrane filter by the application of a vacuum.

In one embodiment, the solution used for treating the cells in step (b) is a washing buffer or a lysis buffer. In an alternate embodiment, if the solution used for treating cells in step (b) is a washing buffer, then after step (d), steps (b) through (d) may be repeated using a lysis buffer as the solution. Optionally, the method will further comprise collecting the lysate from the receiving plate. In one embodiment, the above-described method is used for obtaining mRNA from the cells.

In an alternate embodiment, the multiwell filter plate in combination with the gasket described above is used to obtain mRNA in a method comprising the steps of:

- a) applying cells to the filter plate;
- b) washing the cells with washing buffer;
- c) lysing the cells with lysis buffer; and
- d) collecting the lysate to obtain mRNA.

For purposes of summarizing the invention and the advantages achieved over the prior art, certain objects and advantages of the invention have been described above. Of course, it is to be understood that not necessarily all such objects or advantages may be achieved in accordance with any particular embodiment of the invention. Thus, for example, those skilled in the art will recognize that the invention may be embodied or carried out in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other objects or advantages as may be taught or suggested herein.

Further aspects, features and advantages of this invention will become apparent from the detailed description of the preferred embodiments which follow.

Brief Description of the Drawings

These and other features of this invention will now be described with reference to the drawings of preferred embodiments which are intended to illustrate and not to limit the invention.

Figure 1 shows a filter plate containing the gasket of the present invention. The gasket is placed between the filter plate which has a membrane filter and a receiving plate.

Figure 2 shows a filter plate assembly containing the gasket of the present invention with the application of a vacuum through the porous gasket to facilitate movement of liquid through the filter plate.

Figure 3 shows an alternate embodiment of the presently claimed invention in which the filter plates may be stacked, each filter plate containing a gasket of the presently claimed invention.

Detailed Description of the Preferred Embodiment

Preferred embodiments of the present invention will be explained below. However, the present invention includes various embodiments and is not limited to the preferred embodiments.

A device is described which is designed to eliminate potential cross-contamination between the nozzles and/or undersides of commercially-available or custom-produced filter plates. The device is a porous die-cut or similarly fashioned gasket containing openings corresponding to the nozzles of a filter plate. The gasket 3 is placed on the underside of a filter plate forming a "sandwich" between the filter plate 1 and the receiving plate 2 (see Figure 1). The filter plate contains a membrane filter 4 and wells containing samples in solution 7. The solution passes through the membrane filter 4 and nozzle 5 into the receiving plate 2. When solutions are applied to the top side of a filter plate 1 and exit the nozzles 5 underneath, the gasket 3 will absorb any aerosol or splashing that may occur thus preventing any cross-contamination to adjacent wells 7 of the receiving plate 2.

Movement of the solution may be facilitated by application of either a positive or negative pressure. In a preferred embodiment, a nozzle 6 may be provided for attachment to a vacuum source. The porous nature of the gasket 3 also allows a vacuum to be applied directly through the gasket. This permits a tight sealing "sandwich" to be formed between the filter plate 1, gasket 3, and receiving plate 2, which further reduces potential cross-contamination.

The disclosed gasket comprises a continuous porous plastic membrane, with dimensions similar to a regular microtiter plate, with corresponding holes. In a preferred embodiment, the thickness of the porous membrane is $\frac{1}{16}$ inch to $\frac{1}{2}$ inch. More preferably, the porous membrane thickness is $\frac{1}{32}$ inch to $\frac{1}{8}$ inch. The disclosed gasket is

manufactured from porous polymeric plastic material including, but not limited to, any of the following materials: polypropylene, polyethylene, glass fiber, polyvinylidene fluoride, nylon, nylon-6, polyethersulfone, polytetrafluoroethylene, ethyl vinyl acetate, or other similar materials. Porous polymeric plastic such as hydrophobic polyethylene with median porosity range 45-90 micron materials for the practice of the claimed invention are commercially available from a variety of sources including POREX™ (Fairburn, GA) and FluoroLogic LLC™ (Los Alamitos, CA).

The controlled pore structure within the gasket can have any predetermined average pore size. A range of average pore size from 7-250 microns is preferred. More preferred is a range of average pore size of 45-90 micron. Most preferred is a mean pore size of about 70 micron.

A preferred void volume is at least 35%. A most preferred void volume is in the range of 35-65%.

The gasket material may be prepared using a two-step die-cutting process. In the first step, the virgin gasket material is cut to fit the X-Y dimensions of a microplate footprint. The die has rounded corners which produce the corresponding rounded corners on the gasket. This ensures that no excess gasket is exposed to the surrounding air. Thus, an effective seal between the microplate and the gasket is produced, ensuring sufficient vacuum. The flat surface on either side of the gasket may be sealed, for example, with a polymer or other sealant, to prevent excess air penetration through the gasket. This is desirable if a higher vacuum is needed. In an alternate embodiment, the gasket can remain unsealed to allow greater absorptive ability should unexpected aerosol or droplet contamination occur.

In the second step, a second die is used to punch a desired number of holes in each rectangular die cut piece. In a preferred embodiment, 96 holes are punched. For both steps, the excess material which is cut away from the gasket is removed and discarded.

Alternatively, the gasket can be formed by injection molding with the holes already in place. Injection molding offers potential advantages over die cutting. Injection molding potentially provides a consistently flat surface throughout the gasket to ensure a tight seal, eliminating local areas of gasket compression. Also die cutting can introduce particulate matter onto the surface of the gasket, thus requiring detailed inspection of each part prior to affixing the gasket onto the filter plate. Molding parts can theoretically be accomplished in a Clean Room environment, thus minimizing and potentially eliminating extraneous dust and dirt that is typically found in machine shop or tooling environments. Additionally, molding is more precise than die cutting if the mold is properly constructed. Thus, a much tighter tolerance in variation is achieved from one gasket to the next.

In a preferred embodiment, there are 6-384 holes. In a most preferred embodiment, 96 holes are punched out for a 96-well microplate. The gasket can be manually sandwiched in between the filter plate and the receiving plate. Pores are evenly distributed, consistently sized and form an open-cell tortuous patch throughout the material.

In one embodiment, a cassette is then used to hold the "sandwich" together while the vacuum is being applied. Once the liquid contents of the filter plate have been passed through to the receiving plate, the vacuum is turned off and the sandwich can then be removed from the cassette. In an alternate embodiment, the gasket can be applied to the underside of the filter plate using an adhesive compound. Note that prior to die cutting, the adhesive

sheet material is first applied to the gasket. This prevents any misalignment and/or registration problems were the gasket and adhesive sheet die cut separately.

Various types of appropriate adhesives are known to skilled art workers. One kind of adhesive comprises a sheet of clear flexible plastic material that is sticky on both sides (ECHOtape TM, DC-XS301, 24 inches x 60 yards, Edelstein Diversified, Chino, CA). The sticky sheet is applied to the gasket material and then die cut as described above. A removable sheet is provided on the other side of the sticky sheet and is removed just prior to placing the gasket onto the filter plate. This embodiment provides added convenience for the user in that only two components need to be handled instead of three. That is, filter plate/gasket combination and receiving plate. A cassette would still be used to ensure a tight seal between the gasket and the receiving plate.

The porous nature of the gasket material allows a vacuum to be applied directly through the gasket itself. Thus, no space is required between the gasket and receiving plate. A physically tight seal is achievable and also desirable. To facilitate application of a vacuum, a hollow tube is adhered to the side of the gasket along its entire cross-section perimeter. An opening in the tube allows connection to a vacuum source. When a vacuum is applied, the force of the vacuum permeates and penetrates through the gasket 3 and into the airspace surrounding each nozzle 5 of the filter plate. This facilitates the movement of liquid from the topside of the filter plate 1, through the nozzles 5, past the gasket 3, and into the receiving plate 2 (see Figure 2). The porous nature of the gasket provides sufficient vacuum to facilitate liquid movement, but not overly strong so as to misdirect the liquid flow away from the receiving plate and into the gasket itself.

The thickness of the gasket can vary depending on the nature of the filter plate and length of the nozzles underneath. In one embodiment, the thickness of the gasket varies from $\frac{1}{16}$ inch to $\frac{1}{2}$ inch. In a most preferred embodiment, the thickness of the gasket varies from between $\frac{1}{16}$ inch to $\frac{1}{4}$ inch.

Optionally, a regulator will be provided to control the amount of vacuum applied to the filter plate.

Optionally, a perforated plastic seal is provided for the user to place on the underside of the gasket. The purpose of the seal is to protect the gasket from inadvertent contamination such as placement on a contaminated benchtop. The plastic seal is perforated in either columns or rows allowing the user to expose a subset of the nozzles at any given time. This allows for multiple uses of the filter plate and gasket so that it is not necessary to use all the wells at one time. For example, 8 nozzles of a 96 well filter plate could be used.

The disclosed gasket may be used in combination with a multiwell filter plate, for solid phase synthesis, membrane-based immunological, biological and molecular biological assays, cell harvesting and counting, robotic manipulations, as well as use with a column packing such as silica or polymeric resins, gel filtration beads, and the like. The use of the gasket minimizes and potentially eliminates cross-contamination from nozzle to nozzle. The porosity of the gasket allows a vacuum to be applied directly through the gasket itself. Applications for this device include, but are not limited to, molecular biology research, nucleic acid research (including DNA and RNA research), drug discovery research, combinatorial chemistry research, and other research projects involving the use of microplates and filter plates.

In an alternate embodiment, this invention provides for a multiple vertically-stacked assembly. A tower can be constructed consisting of a collection plate at the base of the tower and alternating assemblies of the disclosed gasket 3 and filter plate (see Figure 3). This configuration provides the user with greater flexibility in introducing different compounds via different filter plates, at the desired time, quickly and easily, simply by turning on the vacuum supply to the appropriate filter plate. As a result, this allows researchers in fields such as combinatorial chemistry to conduct complex meaningful experiments in a rapid and cost-effective manner.

It will be understood by those of skill in the art that numerous and various modifications can be made without departing from the spirit of the present invention. Therefore, it should be clearly understood that the forms of the present invention are illustrative only and are not intended to limit the scope of the present invention.

EXAMPLE 1

Elution of mRNA from BHK-21 and COS-7 cell lines

Two adherent cell lines, BHK-21 (newborn hamster kidney fibroblasts) and COS-7 (African green monkey kidney fibroblasts) were processed using the mRNA *Express* Kit from RNA^{ture}. Between 10,000 and one million cells for each cell line were applied to a multiwell filter plate in combination with a gasket of median porosity hydrophobic polyethylene (mean pore size 70 micron, range 45-90 micron POREXTM). The gasket dimensions were 2 x 4 ¼ x 15 inch. The gasket contained 96 holes. Each hole had a diameter of ¼ inch. Cells were counted with a hemacytometer and were applied in a maximum volume of 200 µl. If necessary, PBS was added to make the sample volumes equal. The cells were washed twice with 100 µl of PBS, lysed with Lysis Buffer, and transferred to a GenePlate[®] following the standard mRNA *Express* centrifugation protocol. Final lysate volumes transferred to the GenePlate[®] were 75 µl.

The mRNA was hybridized to the GenePlate[®] for 90 minutes at room temperature. Elution was performed by adding 80 µl of Elution Buffer to the wells and incubating at 65°C for 5 minutes. The eluted mRNA was transferred to a black, clear bottom microplate and quantitated with the fluorescent RiboGreenTM RNA Quantitation Kit (Molecular Probes) using ribosomal RNA as a standard.

Optimal cell numbers from the cell lines used here are summarized in Table 1.

TABLE 1

Cell Line	Optimal # of Cells	Maximum yield of RNA (ng)
BHK-21	250,000	38.53 ± 5.44
COS-7	250,000	64.13 ± 2.66

EXAMPLE 2

Competitive Quantitative RT-PCR

Competitive reverse transcription PCR (RT-PCR) was used to quantify the expression levels of human α -actin in HS587T cells using messenger RNA isolated with the mRNA *Express* Kit from RNA^{ture}.

Competitive RT-PCR was performed following the recommendations of Freeman, et al. (Freeman et al. (1999) "Quantitative RT-PCR: Pitfalls and Potential." *Biotechniques* 26: 112-115). Primers were chosen to amplify a 420 base pair fragment of the human β -actin gene. A homologous, synthetic β -actin mRNA competitor was constructed with a 50 base-pair deletion. After reverse transcription of this competitor, amplification using the wild-type primers produced a 370 bp fragment.

Messenger RNA was isolated from HS587T human breast cancer cells with the mRNA *Express* Kit. To begin, 4500 HS587T cells were applied to each of six wells on a multiwell filter plate in combination with a gasket of median porosity hydrophobic polyethylene (mean pore size 70 micron, range 45-90 micron POREX™). The gasket dimensions were 2 x 4 1/4 x 1/8 inch. The gasket contained 96 holes. Each hole had a diameter of 1/16 inch. The cells were washed with PBS by centrifugation. 50 μ l of Lysis Buffer containing between 1.3×10^6 and 3.3×10^8 competitor molecules were applied to the appropriate wells and incubated at room temperature for 5 minutes. The lysate was transferred to the GenePlate® by centrifugation (670 x g for 5 minutes) and allowed to hybridize for 15 minutes at room temperature. Following three washes with Wash Buffer, a two-step RT-PCR reaction was performed.

The reverse transcription reaction was conducted at 42°C for 50 minutes in 20 μ l reaction volumes using 100 units of M-MLV reverse transcriptase per reaction. The wells were washed twice with 10 mM Tris, pH 7.5, then subjected to 25 cycles of amplification using a single set of β -actin specific primers in 20 μ l reaction volumes.

The PCR products were analyzed by electrophoresis through a 2% agarose gel pre-stained with ethidium bromide. At the completion of electrophoresis, the gel was photographed with Polaroid Black and White Film Type 665 under UV light. Scanning densitometry was performed using a Personal Densitometer SI from Molecular Dynamics. The ratios of the densities of the competitor and wild type β -actin bands were determined. The logs of these values were plotted against the logs of the number of β -actin competitor molecules originally applied to the wells on the multiwell filter plate.

Messenger RNA isolated by the mRNA *Express* Kit was used successfully with a competitive, quantitative RT-PCR methodology to quantitate the expression levels of human β -actin in HS587T cells. About 4400 copies of β -actin occur in each HS587T cell under the culture conditions employed for this example. This number falls within the reported range of message copies expected for an abundant message in mammalian cells.

WHAT IS CLAIMED IS:

1. A porous gasket sheet having intercommunicating pores in a longitudinal direction, said intercommunicating pores having a size effective to permit air to flow therethrough.

2. The gasket sheet according to Claim 1, which has an edge adapted to be connected to a vacuum source.

5 3. The gasket sheet according to Claims 1 or 2, which is adapted to be secured between a multiwell filter plate and a receiving plate and which has holes corresponding to the wells of the multiwell filter plate.

4. The gasket sheet according to any one of Claims 1-3, which is made of a material selected from the group consisting of polypropylene, polyethylene, polyvinylidene fluoride, polytetrafluoroethylene, nylon, polyethersulfone, and ethyl vinyl acetate.

10 5. The gasket sheet according to any one of Claims 1-4, which has a thickness of $\frac{1}{16}$ inch to $\frac{1}{2}$ inch.

6. The gasket sheet according to Claim 5, which has a thickness of $\frac{1}{16}$ inch to $\frac{1}{4}$ inch.

7. The gasket sheet according to Claim 3, which has 6 to 384 holes.

8. The gasket sheet according to Claim 7, which has 96 holes.

9. The gasket sheet according to any one of Claims 3 or 7 or 8, which has an edge adapted to be
15 connected to a vacuum source, wherein each hole has an inner peripheral edge through which air can be absorbed when the vacuum source is activated.

10. The gasket sheet according to any one of Claims 1-9, which has a range of average pore size of 7 to 250 microns.

11. The gasket sheet according to Claim 10, which has a range of average pore size of 45 to 90 microns.

20 12. The gasket sheet according to Claim 11, which has an average pore size of about 70 microns.

13. A multiwell filter plate in combination with a gasket sheet set forth in any one of Claims 1-12, said gasket sheet corresponding in size and shape to the perimeter of said multiwell filter plate and having a plurality of holes corresponding to the respective wells of the multiwell filter plate, said gasket sheet being adapted to fit between said multiwell filter plate and a receiving plate.

25 14. The multiwell filter plate according to Claim 13, wherein each well of the multiwell filter plate comprises a nozzle for application of a vacuum.

15. The multiwell filter plate according to Claims 13 or 14, wherein each well has a membrane filter at its bottom, and the gasket sheet has an edge adapted to be connected to a vacuum source, wherein the pressure below the membrane filter can be reduced by absorbing air through an inner peripheral edge of each hole when activating the
30 vacuum source.

16. An elution system comprising a multiwell filter plate with a gasket sheet set forth in Claims 13 or 14 and a receiving plate, wherein the gasket sheet is secured between the multiwell filter plate and the receiving plate, each well has a membrane filter at its bottom, and the gasket sheet has an edge adapted to be connected to a vacuum source, wherein the pressure below the membrane filter can be reduced by absorbing air through an inner peripheral
35 edge of each hole when activating the vacuum source.

17. A method of treating cells using the elution system set forth in Claim 16, comprising the steps of:

(a) applying cells to each well of the multiwell filter plate;

(b) treating the cells in each well with a solution;

5 (c) activating the vacuum source to absorb air through each hole of the gasket sheet, wherein the pressure under the membrane filter is reduced; and

(d) enhancing draining the used solution through the membrane filter by the application of a vacuum.

18. The method according to Claim 17, wherein the solution is a washing buffer or a lysis buffer.

10 19. The method according to Claim 17 or 18, wherein the solution is a washing buffer, and after step (d), steps (b) through (d) are repeated using a lysis buffer as the solution.

20. The method according to Claim 19, which further comprises collecting the lysate from the receiving plate.

21. The method according to any one of Claims 17-19, which is used for obtaining mRNA from the cells.

15 22. A use of a multiwell filter plate in combination with the gasket of any one of Claims 1-12 to obtain mRNA comprising the steps of:

e) applying cells to the filter plate;

f) washing the cells with washing buffer;

g) lysing the cells with lysis buffer; and

h) collecting the lysate to obtain mRNA.

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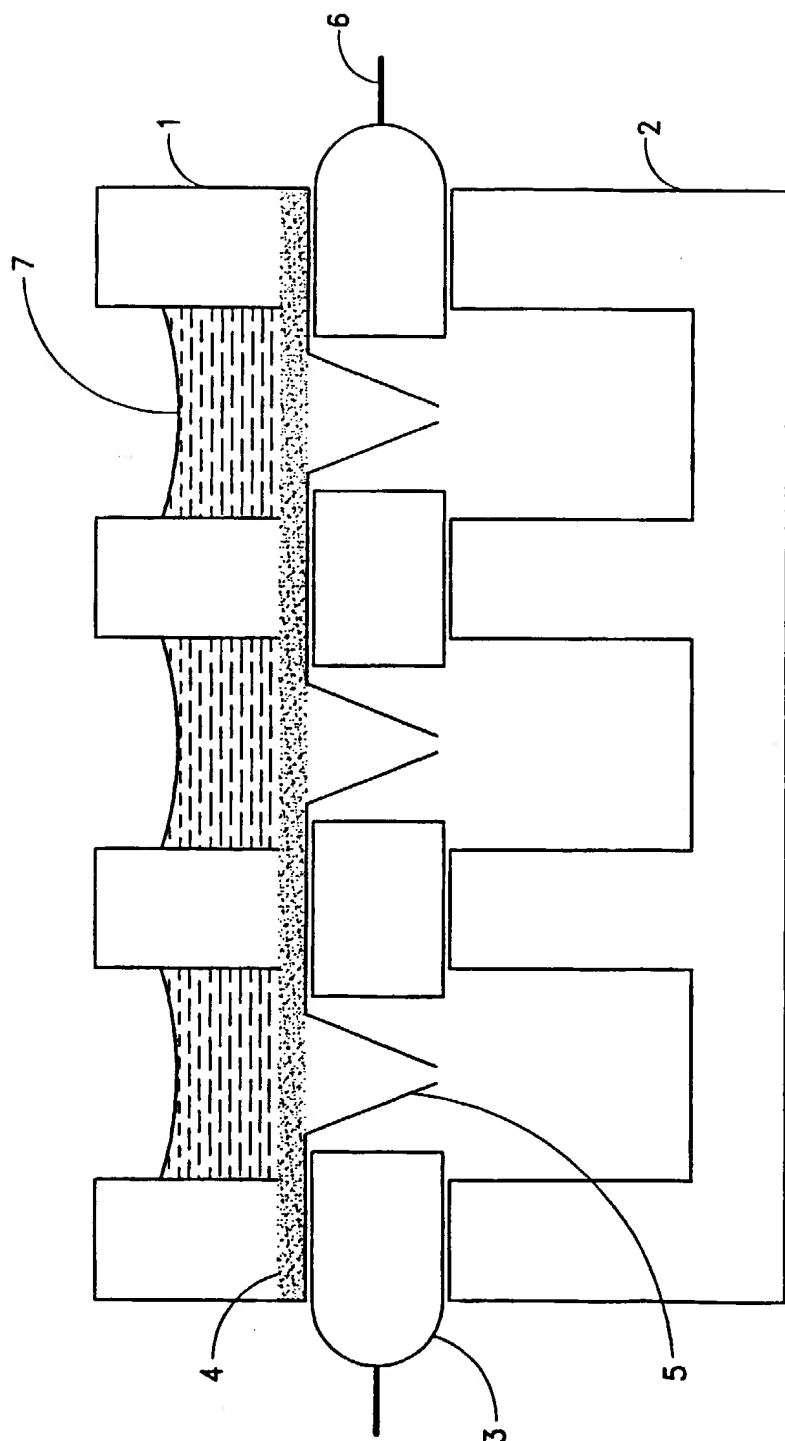


FIG. 1

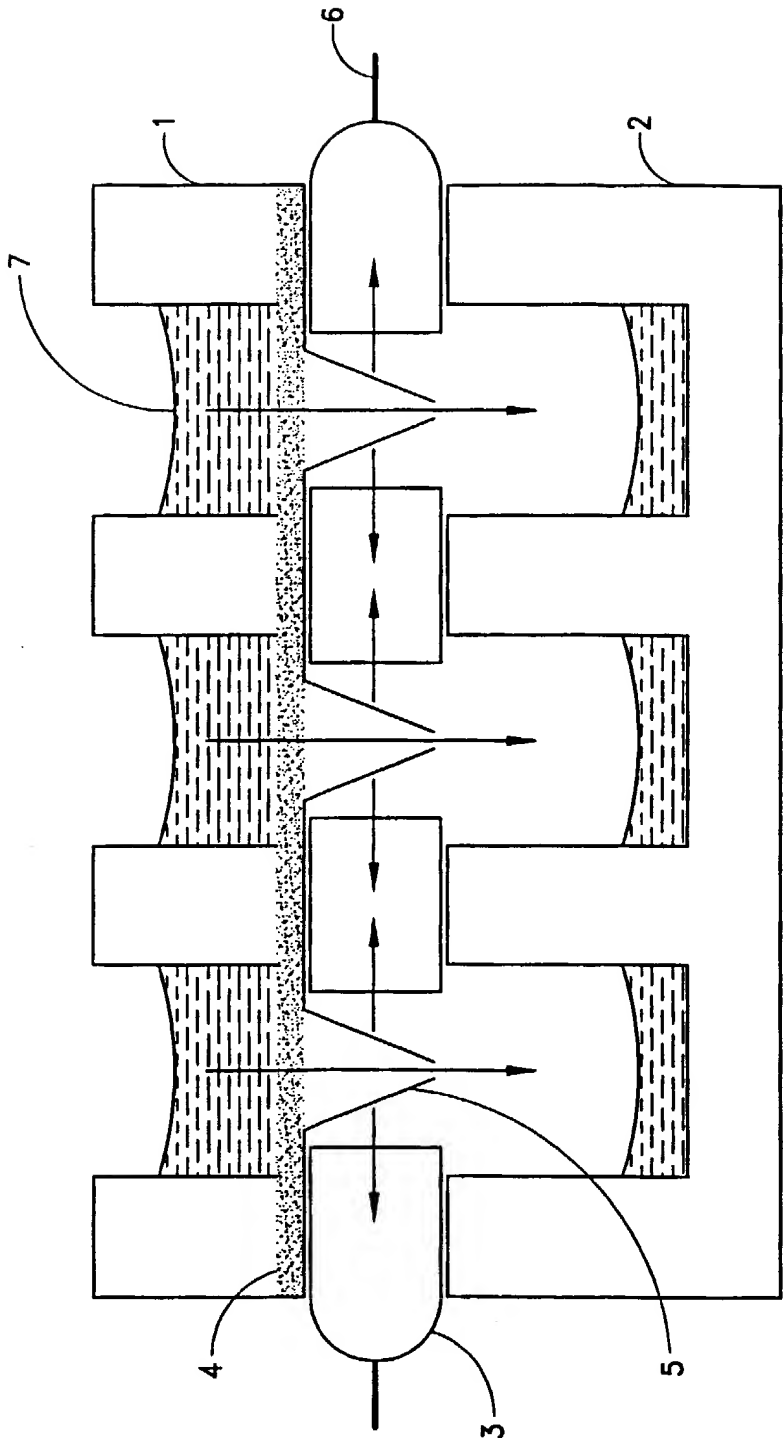
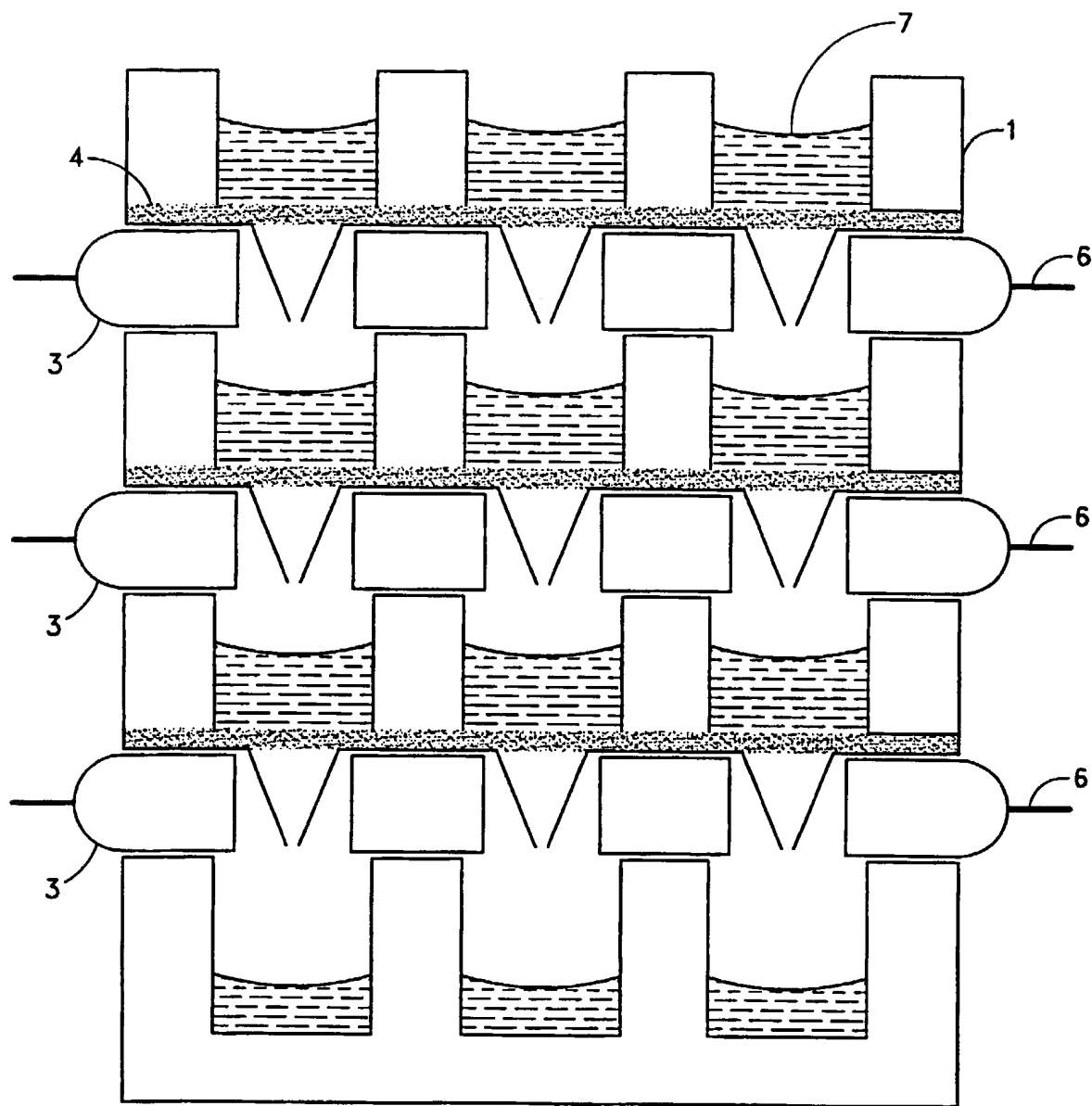


FIG. 2

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*FIG. 3*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/20419**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : B01L 11/00

US CL : 422/101

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/101

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DERWENT, JPAB, EPAB, USPAT

search terms: multi-well filtration, device, apparatus, vacuum, gasket

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,108,704 A (BOWERS et al.) 28 April 1992 (28.04.92, see entire document.	1-3, 7-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

25 SEPTEMBER 2000

Date of mailing of the international search report

02 NOV 2000

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/20419

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-6 and 9-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.